

AWARD NUMBER: W81XWH-16-1-0382

TITLE: A Serum miR Signature Specific to Low-Risk Prostate Cancer

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REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE September 2017		2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2016 - 31 Aug 2017	
4. TITLE AND SUBTITLE A Serum miR Signature Specific to Low-Risk Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-16-1-0382	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Larisa Nonn E-Mail: lnonn@uic.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Illinois College of Medicine, Department of Pathology 840 S. Wood St Chicago, IL 60612				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this new proposal is to develop a miR panel as a serum marker to identify biopsypositive prostate cancer (PCa) patients with a low-risk of harboring aggressive disease. There are several useful pre-treatment risk calculators that use clinical parameters (age, biopsy grade, PSA). These calculators accurately identify high-risk patients defined by clinical parameters. However, there is uncertainty with low/intermediate risk patients with only Gleason grade 3 on biopsy and which of these men require curative treatment. To address this unmet need, we previously identified a serum microRNA (miR) signature that categorized, with extremely high accuracy, a subset of PCa patients with low-risk of harboring aggressive disease. miRs are stable biomarkers resistant to degradation. Our first study used a cohort designed to discover miRs that were differentially present in the pre-surgical sera from a unnatural cohort of 100 PCa patients with either low-grade (Gleason grade 3) or >50% high-grade (Gleason grade 4+5) disease. Using 14 miRs we created a combined "miR Score" which had clear threshold and a negative predictive value of 0.9 to predict the absence of highgrade PCa among the patients. A unique feature of our discovery study that provides confidence in the predictive ability of the miRs is that the entire radical prostatectomy specimen was step-wised sectioned to ensure absence of high-grade tumors in our low-risk group. As well, none of the PCa patients in our high-grade group, which had abundant Gleason 4+5 tumors, had high levels of the miRs in their serum. Thus were able to perfectly categorize any patient with high serum miR levels as low-grade and low-risk.					
15. SUBJECT TERMS Prostate Cancer, serum biomarker, microRNAs					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

The goal of this new proposal is to develop a miR panel as a serum marker to identify biopsy-positive prostate cancer (PCa) patients with a low-risk of harboring aggressive disease. There are several useful pre-treatment risk calculators that use clinical parameters (age, biopsy grade, PSA). These calculators accurately identify high-risk patients defined by clinical parameters. However, there is uncertainty with low/intermediate risk patients with only Gleason grade 3 on biopsy and which of these men require curative treatment. To address this unmet need, we previously identified a serum microRNA (miR) signature that categorized, with extremely high accuracy, a subset of PCa patients with low-risk of harboring aggressive disease. miRs are stable biomarkers resistant to degradation. Our first study used a cohort designed to discover miRs that were differentially present in the pre-surgical sera from a unnatural cohort of 100 PCa patients with either low-grade (Gleason grade 3) or >50% high-grade (Gleason grade 4+5) disease. Using 14 miRs we created a combined “miR Score” which had clear threshold and a **negative predictive value of 0.9** to predict the absence of high-grade PCa among the patients. A unique feature of our discovery study that provides confidence in the predictive ability of the miRs is that the entire radical prostatectomy specimen was step-wised sectioned to ensure absence of high-grade tumors in our low-risk group. As well, none of the PCa patients in our high-grade group, which had abundant Gleason 4+5 tumors, had high levels of the miRs in their serum. Thus were able to perfectly categorize any patient with high serum miR levels as low-grade and low-risk.

The experiments in this study were designed to investigate the serum miRs not only as a biomarker, but also potentially as having biological function in PCa. This is distinct from our discovery study because all of the patients for this proposal will be of low to intermediate-risk. To query the above points this proposal includes two independent aims; the first based on clinical prediction and the second aim queries the origin of the serum miRs. Very little is known about how miRs get into serum (active or passive) and if they have endocrine activity.

2. KEYWORDS: prostate cancer, serum biomarker, microRNAs

3. ACCOMPLISHMENTS:

3A. Major Goals:

Specific Aim 1: To establish the serum miR panel as a pre-treatment predictor of low-risk PCa in independent cohorts of radical prostatectomy patients.			Progress
Major Task 1: Acquire Sera and pathology from PCa patients			
Subtask 1: Obtain IRB approval for study	1-3	Dr. Nonn	100%
Subtask 2: Sera for use in this study will come from sera collected as part of Co-Investigators clinical trial (Fall of 2015 to 2018) N=300	1-24	Dr. Gann	20%
Subtask3: Identification of patients within the sera cohort who will be treated by radical prostatectomy (N=100)	1-24	Dr. Abern	ongoing
Subtask 4: Collect pathology findings from radical prostatectomy specimen (N=100)	1-24	Drs. Gann and Nonn	ongoing
<i>Milestone(s) Achieved: Collection of pre-surgical sera cohort for RNA analysis</i>	24		
Major Task 2: Measure miR panel in PCa patient sera			
Subtask 1: Isolate RNA (ongoing through this time frame)	6-24	Dr. Nonn	0%
Subtask 2: RT-qPCR analysis (ongoing through this time frame)	12-24	Dr. Nonn	0%
<i>Milestone(s) Achieved: miR levels in the pre-surgical serum of PCa patients</i>	30		
Major Task 3: Statistical analysis of serum miR panel to predict risk of favorable pathology			
ongoing through this time frame at 6 month intervals	18-36	Drs. Gann, Nonn and CCTS core	0%

Specific Aim 2: To examine prostatic origin and biological significance of the miRs in the serum panel.			
Major Task 1: Obtain sera and exosomes from PCa patient pre and post radical prostatectomy			
Subtask 1: Identify PCa patients with low-intermediate risk having radical prostatectomy at UIC (N=35*) *75% over estimate to ensure N=20 for study	1-12	Dr. Abern	10%
Subtask 2: Collect leftover fresh serum from those PCa patients pre- and post-surgical blood draws (N=35)	1-12	Drs. Nonn and Abern	10%
Subtask 3: Isolate exosomes from fresh serum(N=35)	1-12	Dr. Nonn	10%
<i>Milestone(s) Achieved: Sera and exosomes for miR analysis</i>	12		
Major Task 2: Identify prostatic miRs in PCa patient serum			
Subtask 1: RNA extraction from the serum and miR profiling by miRNome array	12-18	Dr. Nonn	0%
Subtask 2: statistical analysis to identify miRs differentially detected before and after prostatectomy	18-24	Dr. Nonn	0%
<i>Milestone(s) Achieved: List of miRs in the serum of potential prostatic origin</i>	24		
Major Task 3: Quantify miRs in PCa tissues			
Subtask 1: Create custom TMA of PCa tissues to examine miR expression by Gleason grade (N=60)	1-12	Dr. Nonn	100%
Subtask 2: in situ hybridization for miR detection in TMA (miRs identified in serum as likely prostatic origin)	24-30	Dr. Nonn	25%
Subtask 3: Quantitative analysis of ISH on TMA by Imaging Core (co-Investigator is the Director)	30-36	Dr. Gann	0%
<i>Milestone(s) Achieved: Determine if miRs in the serum are of prostate origin and alter by Gleason grade</i>	36		
Major Task 4: Quantify miRs in serum and prostate exosomes			
Subtask 1: Isolate exosomes from primary prostate epithelial and stromal cells collected from areas of Gleason 3 PCa (N=5 each)	12-18	Dr. Nonn	50%
Subtask 2:RT-qPCR analysis of prostatic miRs in exosomes	18-24	Dr. Nonn	0%
<i>Milestone(s) Achieved: Determine if miRs in the serum are from prostatic exosomes</i>	24		

3B. What was accomplished under these goals?

Major Activities: The key activities of Y1 span both Aim 1 and Aim 2.

Aim 1: We are in the process of completing the serum collection, which will subsequently be used to quantify the serum microRNA panel. We have obtained IRB approval from UIC, the UI Cancer Center and HRPO to initial the studies. Serum collection for Aim 1-Major Task is ongoing and to date we have serum from 40 patients (**Table 1**). However, only two of those patients went on to be treated by radical prostatectomy (RP). RP is required to obtain the endpoint of adverse pathology. This problem is addressed and action has been taken to remedy this for Y2 (see section 8).

Date	Study ID	Serum	Exo-deplete Serum	Exosomes	OncoTypeDX	RP	UIC
7/28/2016	3000	2	Y	Y	LOW		
8/31/2016	3001	2	Y	Y	LOW		
10/13/2016	3003	3	Y	Y	LOW		
10/26/2016	3005	3	Y	Y	LOW		
10/31/2016	3002	3	Y	Y	LOW		
11/2/2016	3004	2	Y	Y	LOW		
11/7/2016	3006	3	Y	Y	LOW		
12/2/2016	4000	4	Y	Y	INT		
12/8/2016	1002	2	Y	Y	LOW		X
12/9/2016	3007	3	Y	Y	LOW		
1/4/2017	1003	4	N	N	LOW		X
12/1/2016	1004	2	Y	Y	INT		
1/25/2017	3013	2	Y	Y	LOW		
2/8/2017	3011	4	N	N	LOW		
2/21/2017	3015	2	Y	Y	LOW		
3/6/2017	3016	2	Y	Y	LOW		
3/6/2017	3014	2	N	N	LOW		
3/14/2017	3019	2	Y	Y	LOW		
3/15/2017	3021	2	Y	Y	LOW		
3/15/2017	3012	2	Y	Y	LOW		
3/16/2017	3020	2	Y	Y	LOW		
3/21/2017	2000	2	Y	Y	INT	X	X
4/6/2017	3022	2	Y	Y	LOW		
5/15/2017	1004	2	N	N	LOW		X
6/19/2017	1006	2	Y	Y	LOW		X
6/21/2017	3024	2	Y	Y	LOW		
7/5/2017	2002	2	Y	Y	INT	X	X
7/11/2017	1005	2	Y	Y	LOW		X
8/3/2017	3025	2	Y	Y	LOW		
8/3/2017	4003	2	Y	Y	INT		
8/17/2017	3026	2	Y	Y	LOW		
8/18/2017	3027	2	Y	Y	LOW		
8/23/2017	3028	2	Y	Y	LOW		
8/25/2017	2003	2	Y	Y	INT		X
9/7/2017	4006	2	N	N	INT		
9/12/2017	3029	2	Y	Y	LOW		
9/15/2017	1008	2	Y	Y	LOW		X
9/20/2017	4006	2	N	N	INT		
9/25/2017	4007	2	Y	Y	INT		
TOTALS	40	40	36	36	40	2	9

Aim 2:

Exosomes in serum. To examine if the prostate is contributing the serum microRNAs, we will measure these microRNA in serum and prostate derived exosomes. For the patient serum, exosomes and exosome-depleted serum have been isolated from serum from 36 patients (**Table 1**). These exosomes of the correct size of 50-100 nm as determined by the Nanocyte (**Figure 1A**) and transmission electron microscopy (TEM) (**Figure 1C**). Another peak is observed at 200 nm (**Figure 1C**), of unknown significance. Although our goal was N=35, only 9 of these patients were from UIC and only 2 have had RP, which we need to acquire post-surgical sera and exosomes.

Exosomes from patient prostate cells. Primary human prostate cell cultures were examined as a source for the microRNAs. We have the unexpected finding that the prostate stroma (PrS) produces 1000-times more exosomes than prostate epithelium (PrE) (**Figure 1B**).

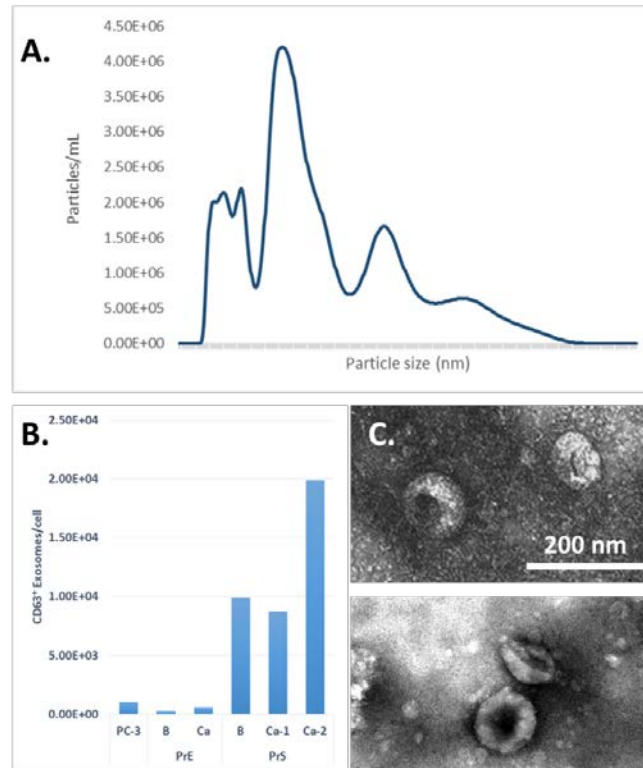


Figure 1. Exosome isolation from patient serum and primary prostate cells. **A**, size analysis of exosomes isolated from patient serum. Size determined on NanoCyte. Peak microvesicles at the size of 50, 100 and 200 nm. **B**, quantitation of exosomes from primary prostate epithelial (PrE) and stroma (PrS) cells by anti-CD63 ELISA. PC3 prostate cancer cells included as positive control. **C**, exosomes imaged by transmission electron microscopy. Representative areas from full-slide image.

microRNA levels in prostate tissues. A tissue microarray (TMA) was constructed from prostate cancer patients. The TMA was completed and has been used to optimize in situ hybridization (ISH) for detection of microRNAs. Since writing the proposal the methodology for ISH has improved and we have successfully used exiqon LNA probes to robustly detect miR-182 in patient samples (**Figure 2**). This miR is not in our prognosis panel, but serves as a positive control for our work as we have previously shown it is present in prostate epithelium by RT-qPCR. Importantly, the miR is present at higher levels, which was anticipated based on our prior findings. The prominent expression in the epithelium compared to the stroma is also evident.

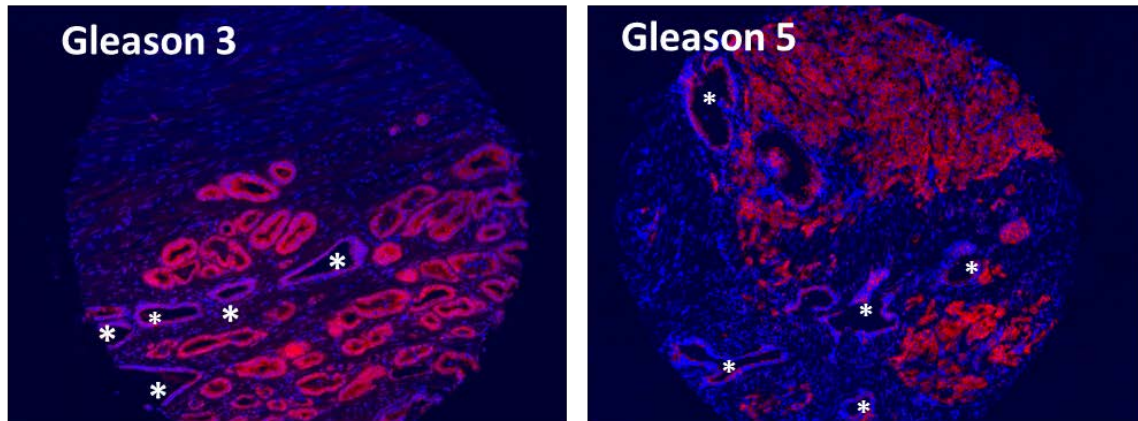


Figure 2. Robust detection of microRNA by in situ hybridization (ISH). ISH using exiqon LNA double labeled DIG probe with anti-DIG and Vector Red. Nuclear counterstain with dapi (blue). Staining is restricted to the epithelium. Areas of benign glands are indicated with *.

Key findings:

- Prostate stroma makes 1000x the amount of exosomes compared to epithelium. This suggests that the stroma may be the major contributor of prostate-derived exosomes into the serum.
- MicroRNAs can be robustly detected in prostate tissues by ISH. Further use of ISH will examine whether the microRNAs in our panel are present in the stroma or epithelium surrounding cancer.

3C. What opportunities for training and professional development has the project provided?

An MD/PhD student, Morgan Zenner, has joined the lab and her effort is 100% to this project. She will be attending the MicroVesicles meeting in October of 2017 and AUA in 2018.

3D. How were the results disseminated to communities of interest?

Nothing to Report

3E. What do you plan to do during the next reporting period to accomplish the goals?

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change: Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Problem: This study is ancillary to the DOD-funded ENACT study (PI: Peter Gann). There were initial delays in IRB approvals that have affected serum numbers for Aim 1. This issues are now resolved and we should reach target patient accrual by 2019.

Problem: The number of patients in ENACT coming from UIC is less than expected patients. As well the number of patients getting subsequent radical prostatectomies is less than expected. These issues impact Aim 2, for which we require pre- and post-surgical sera. Action: We have now tied in to the UIC Prostate Cancer Blood Biorepository (PI: Larisa Nonn) in order to access serum from all UIC prostatectomy patients. This protocol is in place as of August 28, 2017 and we expect to reach our target of 35 patients by Spring of 2018.

Changes that had a significant impact on expenditures

The expenditures are below budget due to the delays in serum collection (described above) and a delay in hiring. Ms. Morgan Zenner is a new MD/PhD student who started full-time in the laboratory of Dr. Larisa Nonn in August 2017. She had previously rotated

in the lab in May 2017 and thus the position was reserved for her. Expenditures for Y2 will be on track.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report.

Significant changes in use or care of human subjects: Nothing to report.

Significant changes in use or care of vertebrate animals: Nothing to report.

Significant changes in use of biohazards and/or select agents: Nothing to report.

6. PRODUCTS: Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Morgan Zenner</i>
Project Role:	<i>Key personnel, MD/PhD student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	<i>Ms. Zenner started in the Nonn Lab in August 2017 and devotes 100% effort to this project.</i>
Funding Support:	<i>none</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: not applicable

Quad Charts: not applicable

9. APPENDICES: none